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Report

A Potentially Fatal Mix of Herpes in Zoos

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Summary

Pathogens often have a limited host range, but some can opportunistically jump to new species. Anthropogenic activities that mix reservoir species with novel, hence susceptible, species [1] can provide opportunities for pathogens to spread beyond their normal host range. Furthermore, rapid evolution can produce new pathogens by mechanisms such as genetic recombination [2]. Zoos unintentionally provide pathogens with a high diversity of species from different continents and habitats assembled within a confined space. Institutions alert to the problem of pathogen spread to unexpected hosts can monitor the emergence of pathogens and take preventative measures [3]. However, asymptomatic infections can result in the causative pathogens remaining undetected in their reservoir host. Furthermore, pathogen spread to unexpected hosts may remain undiagnosed if the outcome of infection is limited, as in the case of compromised fertility, or if more severe outcomes are restricted to less charismatic species that prompt only limited investigation. We illustrate this problem here with a recombinant zebra herpesvirus infecting charismatic species including zoo polar bears over at least four years. The virus may cause fatal encephalitis and infects at least five mammalian orders, apparently without requiring direct contact with infected animals [4–8].

Results

Virus Identification

In June 2010, two cohoused polar bears (*Ursus maritimus*), a threatened species, suffered epileptiform seizures at the Zoological Gardens Wuppertal, Germany. The 20-year-old female, Jerka, presented symptoms first and died 8 days after the onset of clinical signs (see Movie S1 available online). Necropsy indicated moderate to severe nonsuppurative encephalitis and gliosis of unknown etiology as the cause of death. The perivascularly accentuated inflammation consisted of lymphocytes and plasma cells. The lesions were consistent with virus infection, but inclusion bodies that are associated with some viral pathogens, including herpesviruses, were not identified. The 16-year-old male, Lars, survived after medical intervention, which included intravenous administration of a hypertonic electrolyte solution and diazepam to prevent seizures (Movie S1). It took several weeks for him to fully recover.

Faced with many potential causative agents, we applied PCR targeting eight plausible encephalitis pathogens (Experimental Procedures) and high-throughput DNA microarrays (ViroChip) to extracted DNA and RNA from Jerka's brain [9]. The ViroChip can test for the presence of several thousand known DNA and RNA viral sequences in a single assay but is biased toward human viruses and is less sensitive than other methods such as PCR or deep sequencing [10, 11]. PCR is very sensitive but prone to false negatives if the sequence targeted by the primers does not match perfectly. The only pathogen detected in Jerka was a virus related to equid herpesvirus 1 (EHV1) [12]) identified by quantitative PCR. Additional PCR assays and the ViroChip did not detect other candidate pathogens (Table 1). Western blot analysis using antibodies against the EHV1 IR6 protein [13] supported the finding of herpesvirus nucleic acids and demonstrated protein expression exclusively in Jerka's brain (Figure 1; Supplemental Experimental Procedures).

Virus Phylogenetics

Equid herpesvirus 9 (EHV9), a virus thought to have originated in the plains zebra (*Equus burchelli*) and that is closely related to EHV1, has been known to cause fatal encephalitis in polar bears [14, 15]. EHV1 related to domestic horse strains has also been shown to cause fatal encephalitis in black bears (*Ursus americanus*) [8]. To determine the identity of the viral strain from Jerka, we sequenced portions of six genes, namely *UL49.5*, 484 bp; *UL45*, 667 bp; *gC* (*UL44*), 1,523 bp; *Pol* (*UL30*), 810 bp; *gB* (*UL27*), 1,140 bp; and *IR6*, 342 bp, and phylogenetically compared the obtained sequences to known EHV1, EHV9, and more distantly related EHV4 sequences (alignments available in the Supplemental Data Sets). All amplicons were directly sequenced and cloned into standard plasmid vectors. Multiple individual clones per amplicon were also sequenced to confirm the sequences obtained from direct sequencing. The sequencing analyses provided no evidence for coinfection with more than one EHV strain.

Jerka's sequences clustered with horse (*Equus caballus*) EHV1 strains but were divergent (1%–3%) at the nucleotide level and formed a sister lineage for the *UL49.5*, *gC* (*UL44*), *Pol* (*UL30*), and *IR6* genes (Figure 2A). The *UL45* and *gB* (*UL27*) gene sequences and part of *Pol* (*UL30*) were nearly identical to a distinct EHV1 strain of plains zebras, designated here as zebra EHV1, which is clearly different from the strain that caused fatalities in black bears (Figure 2A, *UL45* tree) [6]. The remaining genes that we sequenced from Jerka have not been determined for the zebra EHV1 strain. Many of the substitutions were nonsynonymous and changed amino acid sequences when compared to the reference horse EHV1 strain Ab4 (Table S1). Zebra EHV1 is widespread in zoo zebras and is known to infect and cause encephalitis in four other orders of mammals following experimental inoculation or after natural infection in zoos (Figure 2) [4–7].

Surprisingly, the *Pol* (*UL30*) gene was a recombinant between EHV1 and EHV9, with the 5′ portion of the amplicon being EHV1-like, the middle being EHV9-like, and the last

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Table 1. Serological and qPCR Results for the Eleven Polar Bears in This Study

Polar Bear	Sample	EHV1 Diagnostic	Result
Jerka	blood[a]	SNT	<1:4
	brain	qPCR gB	Ct 30.2
	brain	qPCR gC	Ct 31.5
	brain	PCR	DNA sequences
	brain	western blot	IR6 positive
	liver	western blot	IR6 negative
Lars	serum	SNT	<1:4
	feces	qPCR gB	Ct 38.5
	feces	qPCR gC	Ct 39
	saliva	qPCR gB	Ct 38
	saliva	qPCR gC	Ct 37.4
Struppo	serum	SNT	1:16
	brain	qPCR gB	negative
	brain	qPCR gC	negative
	brain	western blot	IR6 negative
	blood	PCR	DNA sequences
Knut	blood[a]	SNT	<1:4
	brain	qPCR gB	negative
	brain	qPCR gC	negative
Anton	serum	SNT	<1:4
	blood	qPCR gB	negative
	blood	qPCR gC	negative
Sonja	serum	SNT	<1:4
	brain	qPCR gB	negative
	brain	qPCR gC	negative
	brain	western blot	IR6 negative
Felix	serum	SNT	<1:4
Anuschka	serum	SNT	<1:4
Nanuk	serum	SNT	<1:4
Anastasia	serum	SNT	<1:4
Anja	serum	SNT	<1:4

Serum neutralization test (SNT) and quantitative PCR (qPCR) results are indicated for each bear. The two bears from which DNA sequences were obtained are also indicated. qPCR results are shown as threshold cycle (Ct) values with the number provided. Brain qPCR results are based on 200 ng of extracted DNA; feces and saliva qPCR results are based on DNA extracted from 16.6 μl of swabs.

[a]SNT from Jerka and Knut performed from blood as serum could not be isolated.

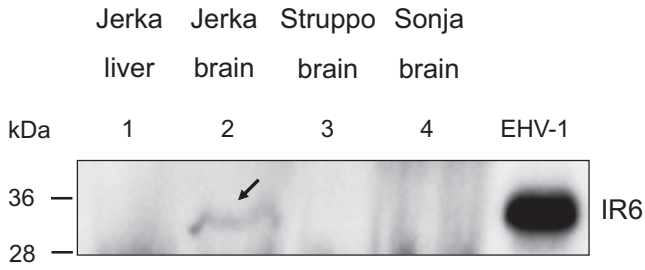

Figure 1. Detection of EHV1 *IR6* Gene Product from Polar Bear Tissue Samples

Samples were homogenized and lysed in RIPA buffer. The lysates were separated by 12% SDS-PAGE, transferred to polyvinylidene fluoride membranes, and analyzed by western blot using an IR6-specific antibody [13]. Lysates of RK13 cells infected with EHV1 Ab4 strain were included as a positive control. Primary rabbit anti-IR6 polyclonal antibody was used at a dilution of 1:10,000, followed by goat anti-rabbit IgG conjugated with horseradish peroxidase. The arrow indicates the *IR6* gene product detected in Jerka's brain (lane 2) but not in other tissues (liver, lane 1) or other bears (Struppo, lane 3; Sonja, lane 4).

110 bp again being EHV1-like (Figures 2B and S1B; Experimental Procedures). As a consequence, Jerka's sequences showed variable affinity to EHV1 and EHV9 as a function of the portion of *Pol* (*UL30*) sequenced (Figure S1A). Although the second recombination breakpoint within *Pol* (*UL30*) did not reach statistical significance, the sequences downstream of position 700 in *Pol* (*UL30*) and sequences upstream and downstream of *Pol* (*UL30*) clustered exclusively with EHV1, including *gB* (*UL27*) found in close proximity on the genome (Figure 2). Thus, both recombination breakpoints are probably within the *Pol* (*UL30*) gene itself. Although a mixture of two EHV strains, the complete 810 bp amplicon sequence showed a stronger overall phylogenetic affinity with EHV1 [Figure 2A, *Pol* (*UL30*) tree], results consistent with the sequence analyses of other genes that also point to EHV1 as the most likely candidate agent for the clinical condition seen in Jerka.

The Distribution of Zebra EHV1 in Zoo Polar Bears

To assess the overall prevalence of the identified virus in our collection of captive polar bear samples, we performed serum neutralization tests against EHV1 and quantitative PCR on samples from 11 unrelated polar bears from five different zoos (Table 1). Including Jerka and Lars, 3 of 11 (27%) of the bears were positive, with recent samples tested from Lars being at the limit of detection (Table 1; Experimental Procedures). Sequencing a 184 bp *Pol* (*UL30*) fragment from the blood of an asymptomatic seropositive bear, Struppo, who died from glomerulonephritis in 2006 and was never cohoused with Jerka or Lars, showed that he was infected with a zebra EHV1 identical to Jerka's for the portion sequenced (Figure S1A). In the case of Jerka, the virus was only detected in brain and was associated with acute lethal infection. Struppo was only positive in blood, suggesting that the route of infection may have been different.

Discussion

Zebra EHV1 likely caused Jerka's death within 8 days, may have sickened Lars, and independently infected Struppo asymptomatically four years earlier in another zoo. Struppo was never cohoused with either Jerka or Lars, and thus the infections are almost certainly independent events. Intensive and expensive high-throughput methods were required to identify one pathogen and rule out many others. Zebra EHV1 represents the second EHV associated with fatality in captive polar bears. EHV9, which may also be a zebra-derived virus, has also been associated with captive polar bear fatalities [14, 15]. Such distantly related hosts are unexpected for herpesviruses, which typically are highly adapted to one particular host. In fact, among the closest relatives of EHV1 and EHV9 in the genus *Varicellovirus*, only pseudorabies virus, a pathogen of pigs that causes morbidity and mortality in bovines and domestic cats and dogs, is known to have jumped to other species under natural conditions [16, 17]. Our phylogenetic analyses suggest that the virus we detected in the polar bears originated in zebras, where zebra EHV1 recombined with EHV9. It remains unclear what the frequency of infection of zebras with EHV1 and EHV9 is in captivity. Such information, however, is critical for the appropriate epidemiological management of polar bears and other threatened species in captivity.

Recombination is not uncommon for herpesviruses, but it usually does not result in the acquisition of new hosts. It is unclear at present where the recombination occurred,

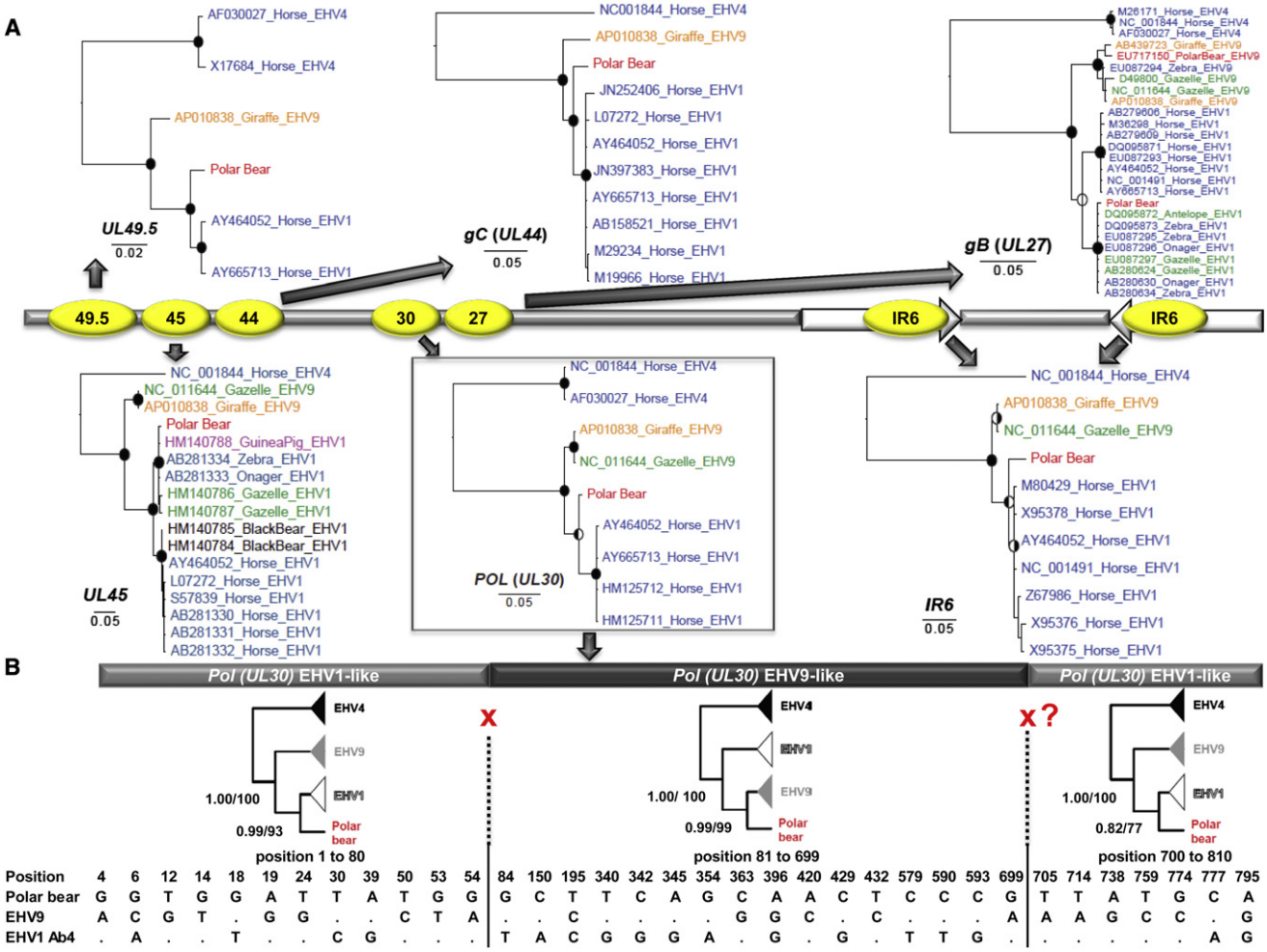

Figure 2. Phylogeny of Polar Bear EHV1

(A) Results of the phylogenetic analysis of polar bear EHV sequences. The schematic of the EHV genome is shown in the middle of the figure indicating relative gene positions designated by their UL number where applicable. *IR6* is repeated twice, but we found no evidence for differences between the two copies in our sequence data. An estimated phylogeny is shown for each gene sequenced. Each database-extracted sequence is denoted by GenBank accession number, species from which the sequence was isolated, and viral strain, separated by underscores. Polar bear sequences from this study and an EHV9 sequence determined previously [15] are highlighted in red. Sequences derived from domesticated and wild horse are in blue, giraffe (*Giraffa camelopardalis*) in orange, guinea pig (*Cavia porcellus*) in purple, antelope (*Antilope cervicapra*) and gazelles (*Eudorcas thomsoni*) in green, and black bear (*Ursus americanus*) in black. Node support is indicated by black semicircles for >90% support or white semicircles for <90% (but not <50%) support. At each node of interest, the left semicircle represents the posterior probability, and the right semicircle represents the maximum-likelihood bootstrap support. The *Pol* (*UL30*) phylogenetic tree is highlighted and connected to (B) to indicate its further recombination analysis.

(B) Detection of recombination between EHV1 and EHV9 in the *Pol* (*UL30*) gene. EHV1-like sequences are in white, and EHV9-like sequences are shaded gray. The diagram at top indicates that the genes upstream and downstream of *Pol* (*UL30*) show greatest affinity to EHV1, whereas *Pol* (*UL30*) varies in its affinity across the sequence determined in this study. The middle part of the figure demonstrates that within *Pol* (*UL30*), the first 80 bp amplified show a strong phylogenetic affinity to EHV1, whereas positions 81–699 demonstrate affinity to EHV9. Positions 700–810 show greatest affinity to EHV1, although they contained only six phylogenetically informative sites. As a consequence, only the recombination breakpoint at position 80 was statistically significant (see Experimental Procedures and Figure S2 for the phylogenetic analysis on which the trees in the figure are based). No statistically significant result for a second breakpoint was obtained, as indicated by the red question mark. However, the phylogenetic affinity of the final 110 bp sequenced was to EHV1, and all sequences downstream of *Pol* (*UL30*) were more closely related to EHV1 than to EHV9, suggesting that the second breakpoint is contained within *Pol* (*UL30*). Posterior probabilities and likelihood bootstrap support are shown at each node. The bottom of the figure shows the nucleotide differences of the polar bear sequence relative to EHV9 and reference EHV1 strain Ab4. Identical sequences are represented by dots, and differences are indicated by the base substitution relative to polar bear.

although we surmise it could have occurred in African-born or zoo zebras. Similarly unclear is whether the newly recombined virus has a broadened host range and/or greater pathogenic potential. The latter is a distinct possibility because *Pol* (*UL30*) is one factor that determines neurovirulence of EHV1 in horses [18, 19], and recombination of EHV1 and EHV9, identified in polar bears, was within *Pol* (*UL30*). The findings suggest that *Pol* (*UL30*) may play an important role in the outcome of infection of nonequine species as well. It is noteworthy that EHV1 infections of nonequine species, such as llamas and alpacas, can have devastating neurological consequences characterized by involvement of a variety of cell types and that often result in death [20]. Whereas two of the polar bears, Jerka and Lars, developed acute neurological

symptoms consistent with observations in nonequids, Struppo remained asymptomatic, and zebra EHV1 was detected in blood and not brain in his case. We speculate that these different manifestations and disease outcomes are a result of different routes of virus entry or virus dose. Jerka and Lars could have been infected directly with large amounts of virus via the conjunctiva and eye or the olfactory nerve, whereas Struppo may have encountered lower doses of virus via a different route in the periphery.

The mode and source of transmission of the newly discovered recombinant virus remain unknown, however. The polar bear enclosure where Jerka and Lars were housed is 68 m away from the zebra enclosure and 340 m from the guinea pig enclosure (another species susceptible to zebra EHV1). The polar bear caretakers were also responsible for guinea pigs in the Zoological Gardens Wuppertal at the time of the outbreak. It is conceivable, therefore, that personnel may present a mode of transmission, as might the ubiquitous presence of rodents in zoos that could be responsible for transmission of the infection from one enclosure to the next. Future research will consider the epidemiology of zebra EHV1 in the captive zebra population and in other species with known and unknown susceptibility to zebra EHV1.

In conclusion, we find that the zebra EHV1 strain reported here may critically threaten zoo animals because of its apparent temporal and geographical spread in the zoo bear population and its ability to infect a wide range of taxa. The recombinant strain apparently spreads without obvious or known direct contact between infected animals. Because its mode of transmission is unknown, control will be difficult. Asymptomatic infection of one bear with the recombinant zebra virus suggests that the host range might be even broader than described. The threat that the virus poses is particularly acute because several zebra species are widespread in zoo collections and because the closely related EHV9 has also jumped to polar bears. EHV9 is also associated with fatality in polar bears [14, 15] and other ubiquitous zoo species such as gazelles [21], which might become new reservoirs and facilitate transmission of viruses. Our analysis reinforces the potential of zoos for disease outbreak monitoring [3, 21] and indicates that the spread of opportunistic and potentially deadly pathogens among captive, nonsympatric threatened species can endanger the conservation mission of zoos.

Experimental Procedures

ViroChip and Pathogen PCRs

Polar bear samples examined in this study are described in the Supplemental Experimental Procedures and Table S1. The ViroChip is a DNA microarray used to detect known viruses and to discover newly emerging ones [10, 22]. The microarray has oligonucleotides representing all known viral classes. ViroChip analysis was performed essentially as previously described [11]. It should be noted that few EHV1- or EHV9-related oligonucleotides are present on this array system, and sensitivity is far lower than PCR or deep sequencing methods [11]. The method is a general screen, but not sensitive enough to definitively exclude pathogen presence in a sample, especially if it is highly underrepresented relative to host DNA or if there are mismatches between sequences in the sample and those on the chip. Experimental details are provided in the Supplemental Experimental Procedures.

PCRs for specific encephalitis-causing pathogens were carried out on DNA or RNA depending on the viral genome using primers for bornavirus [23], adenovirus [24], West Nile virus [25, 26], tick-borne encephalitis [27], rabies [28], parvovirus [29], and canine distemper virus [30, 31] as described in the cited references, with the exception that MyTaq HS from Bioline was used. In the rare cases where a PCR product was detected, such as for canine distemper virus, the product was Sanger sequenced (StarSEQ, Mainz, Germany) with the forward and reverse primers used in the PCR. None of the sequences was of viral origin. PCRs for EHV1 are described in detail in the Supplemental Experimental Procedures.

EHV1 Serum Neutralization Assay

Serum neutralization assays were performed as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [32], with minor modifications. Sera were inactivated for 30 min at 56°C, and 25 μl of sera was diluted with an identical volume of minimal essential medium (MEM) supplemented with 5% fetal bovine serum (FBS). Duplicates of the sera were diluted in 96-well plates in log2 steps in MEM-FBS before addition of 100 tissue culture infectious doses 50% ($TCID_{50}$) per well of EHV1 strain RacL11 in 25 μl of MEM-FBS. Plates were incubated at 37°C for 60 min before addition of 5×10^4 RK13 cells in a total volume of 100 μl. Cytopathic effects were assessed after 5 days of incubation at 37°C by crystal violet staining. A negative control serum (titer < 4) and a positive control serum (titer = 512) were included in each plate. The titer of each serum was determined as the reciprocal of the highest dilution at which the monolayer was intact in both duplicate wells. Plates were included in the assessment only when negative and positive control sera showed the expected values. Results for the 11 bears tested in this study are shown in Table 1.

Recombination and Phylogenetic Analyses

Recombination analysis was conducted using the distance-based method implemented in Recombination Analysis Tool 1.0 [33]. The size of the sliding window was set to 10% of the alignment length, with increments of half this length. In order to improve estimates of genetic distances between pairs of sequences, GenBank sequences with extensive missing data were removed prior to recombination analysis.

Phylogenetic analysis of each gene was conducted using maximum-likelihood and Bayesian methods. Substitution models were selected using the Bayesian information criterion. Maximum-likelihood analyses were conducted with MEGA 5 [34] using a heuristic search with the nearest-neighbor-interchange algorithm. Phylogenetic support was estimated using 1,000 bootstrap replicates.

Bayesian phylogenetic analysis of each gene was performed using MrBayes 3.2 [35]. Posterior estimates of parameters, including the tree topology, were obtained using Markov chain Monte Carlo (MCMC) sampling. Samples were drawn every 2,000 steps over a total of 20 million MCMC steps, with the first 10% of steps discarded as burn-in. Two independent runs were performed, each with one cold and three heated chains. Sufficient sampling from the stationary distribution was checked by inspecting the standard deviation of split frequencies, and additional MCMC simulations were conducted where necessary.

Accession Numbers

Polar bear sequences determined in this study were deposited in GenBank with accession numbers JQ692311–JQ692316.

Supplemental Information

Supplemental Information includes one figure, one table, Supplemental Experimental Procedures, Supplemental Data Sets, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.07.035.

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